The diet of red-throated divers (*Gavia stellata*) overwintering in the German Bight (North Sea) analysed using molecular diagnostics

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Abstract

In Europe, the German Bight is one of the most important non-breeding areas for protected red-throated divers (*Gavia stellata*). It is unclear what attracts the birds to this area, especially as the food composition of seabirds outside the breeding season is notoriously difficult to study. To obtain information on prey species composition of red-throated divers in this area, faecal samples from 34 birds caught alive were analysed using DNA metabarcoding. Prey DNA was detected in 85% of the samples with a mean number of 4.2 ± 0.7 taxa per sample (n=29). Altogether we found a broad prey spectrum with 19 fish taxa from 13 families dominated by five groups: clupeids, mackerel, gadoids, flatfish and sand lances with clupeids being the most frequently detected prey.

Our results indicate that red-throated divers are generalist opportunistic feeders in the German Bight, but pelagic schooling fish that aggregate at frontal zones and have a high energetic value might be favoured. Atlantic mackerel appears to be a more important prey for red-throated divers in this area than previously thought.

The precision achievable using metabarcoding has revealed a number of prey species that are consumed by red-throated divers in the German Bight, which helps to explain the selection of this area by divers in winter and spring.

Key words: Diet composition, DNA Metabarcoding, Next Generation Sequencing, North Sea, Red-throated diver/loon, Site selection

Introduction

Understanding resource utilisation is fundamental for managing wildlife populations. Data on diet composition and feeding strategies are essential for understanding habitat selection and for predicting the ecological consequences of habitat change (Davoren et al. 2003). Predator abundance is often regulated by bottom-up effects of prey abundance (Engelhard et al. 2013). Thus, the availability of prey may affect not only predator distribution and abundance but also foraging strategies (Fauchald et al. 2011; Lynam et al. 2017).

Diet composition of seabirds outside the breeding season, when they remain at sea, is notoriously difficult to study. This is especially true for protected species where only non-invasive methods are applicable. In the past, various techniques have been developed to analyse seabird diet. These include visual observations, morphological identification of regurgitates or gut contents, or biochemical methods such as the analysis of fatty acid and stable isotope concentrations (Barrett et al. 2007; Meier et al. 2017; Quillfeldt et al. 2017; Quinn et al. 2017). A highly efficient alternative approach is to use DNA metabarcoding (Deagle et al. 2005, 2007; Pompanon et al. 2012; Vesterinen et al. 2013; Alonso et al. 2014). This involves amplification of DNA from faecal material and assignment of taxonomical information using Next Generation Sequencing (NGS) and DNA barcode databases.

Our study focused on the prey spectrum of the red-throated diver (*Gavia stellata*), a protected marine bird species, in its wintering and spring staging areas in the German Bight (eastern part of the North Sea). During the non-breeding season about 84,200–186,000 individuals stay in the Baltic Sea, the North Sea and the NE-Atlantic (BirdLife International 2018; Dierschke et al. 2012). Around 20% of the NW-European wintering population occurs in the German Bight (Dierschke et al. 2012; Garthe et al. 2007; Mendel et al. 2008) classifying it as an internationally important staging area for these birds, especially in spring before migration starts (Garthe et al. 2012, 2015). To date three studies have been published on the prey composition of non-breeding red-throated divers in the North Sea and the Baltic Sea, which analysed gut contents using morphological tools (Table 1). However, information is not available from the German Bight (Fig. 1). Red-throated divers feed on a wide range of fish species and, given that the energy content of prey fish varies with size and season, they appear to choose prey of high energetic value (Pedersen and Hislop 2001) like gadoids (Madsen 1957) or clupeids (Durinck et al. 1994; Guse et al. 2009). Additionally cephalopods were found in one of these studies (Durinck et al. 1994) in four of eight birds. Small specimens of polychaetes, crustaceans, copepods, bivalves and gastropods were reported in all studies

although these were considered to be secondary prey (i.e. prey in the guts of the fish eaten by the divers). The German Bight is characterised by an estuarine frontal system, created by the Jutland costal current (JCC) that is primarily driven by discharges from the Elbe river and other rivers further south (Skov and Prins 2001). Red-throated divers have been shown to concentrate at the productive frontal zone, where prey fish aggregate (Skov and Prins 2001). The area is also suitable for the development of offshore wind farms as it has extensive areas of shallow waters (< 40 m). To date, 17 wind farms have been installed in German North Sea waters. Thus, there is potential overlap between offshore wind farm sites and the preferred habitat of non-breeding red-throated divers (Garthe et al. 2015; Heinänen et al. unpubl data). Red-throated divers have been shown to strongly avoid both shipping traffic and wind farms (Garthe and Hüppop 2004; Bellebaum 2006; Petersen et al. 2006; Dierschke et al. 2006, 2012; Mendel et al. 2019; Heinänen et al. unpubl data; Burger et al. unpubl data). To understand the environmental importance of the German Bight for red-throated divers, to assess the possible impacts arising from displacing divers from substantial parts of their staging areas, and to analyse whether alternative staging areas might be available, it is crucial to understand what resources these birds rely on.

In this study we had the unique opportunity to collect a small number of faecal samples from red-throated divers captured in the German North Sea in 2015 and 2016 in both winter and spring. We applied DNA metabarcoding as a non-invasive technique to analyse diet composition, and thus to provide a detailed overview of recent meals of these birds in the German Bight. Specifically, we aimed to document the diversity of prey species eaten by the birds in this particular staging area when red-throated diver abundance is highest. Additionally, we aimed to compare data for two consecutive sampling years to determine if the prey species consumed is consistent between years. By comparing dietary data with published data on local fish distribution, we aimed to determine whether the abundance and distribution of prey fish correlate with red-throated diver diet and how this may help to explain red-throated diver distribution.

Methods

Sample collection and study site

This dietary study was part of a satellite telemetry project on red-throated divers. A total of 36 red-throated divers were captured in March and April 2015 and in February and March 2016 in the German Bight (Fig. 1). Sampling was focused on late winter and spring when red-

throated diver abundance is highest in the German Bight (Mendel et al. 2008; Dierschke et al. 2012; Garthe et al. 2015). The capture area was approximately 30 km offshore in water depths of around 20 m, which is approximately in the centre of the staging area for red-throated divers (Fig. 1). Birds were captured from a rigid inflatable boat using a hand net and the "night lighting technique", where the sea is searched for resting divers with a spot light. If a bird is sighted, it often becomes disoriented by the bright light and can be captured with a net (Whitworth et al. 1997; Ronconi et al. 2010). In 2015 captured birds were kept in boxes for an average time of 18.3 h (min 6.3 h, max 27 h) and in 2016 for an average time of 9.2 h (min 7 h, max 13 h). After release the boxes were searched for scat. The boxes were cleaned and disinfected after every use with bleach (1% hypochlorite solution), water and ethanol (70%) to prevent cross contamination. During the two field seasons a total of 34 faecal samples were collected (2015 n = 15; 2016 n = 19, Table 2). Samples were preserved in absolute ethanol and stored at -20°C until further analysis.

DNA extraction

Faecal DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturers protocol with the following modifications: (i) the samples were resuspended in the storage ethanol by vortexing before moving 200 µL of the ethanol-scat slurry to a new clean 2 ml Eppendorf tube and centrifuging for 30 s at 4000 x g (Deagle et al. 2005); (ii) the lysis step was extended by adding 1.4 mL Buffer ASL instead of 1.6 mL to each sample and incubating at 70 °C for 10 min and then for 1.5 h at room temperature to improve lysis output; (iii) the digestion step was extended by adding 20 µl instead of 25 µl proteinase K and incubating samples at 70 °C for 30 minutes prior to an increased incubation time at a lower temperature (56 °C for 1.5 h). All remaining steps followed the manufacturer's instructions except that buffer volumes were cut down to reduce risk of cross contamination by minimizing the number of pipetting steps and by reducing the volume of liquid loaded into spin columns and tubes (Deagle et al. 2005). The final elution step used a total elution volume of 100 µl (as recommended by the manufacturer`s protocol), but was divided into two steps with each elution using 50 µL Buffer AE.

Primer design and preparation for sequencing

Three separate PCR primer pairs were used to comprehensively target all the major potential prey species of red-throated divers in this area (Table 3). These prey species are widespread in

131 the North Sea and were informed by previous diet studies on red-throated divers (Table 1;
132 Madsen 1957; Durinck et al. 1994; Guse et al. 2009).

133 Primers for each prey group were tested *in silico*, using ClustalX 2 (Larkin et al.2007) and
134 MEGA7 (Kumar et al. 2016). Conserved primer binding sites were tested against a DNA
135 barcode database of barcode-sequences extracted from GenBank. Sequences of 16S DNA of
136 28 representative fish species from 7 orders and 15 families as well as 12 cephalopod species
137 from 5 families were aligned for these tests. For crustaceans COI barcode sequences of
138 potential prey species from 6 orders and 8 families of shrimp and krill were aligned and
139 tested. Furthermore primers for each prey group were tested *in vitro* on DNA from tissue
140 samples of corresponding potential prey species occurring in the German Bight (clupeids,
141 perciformes, gadoids, flatfish, octopus, squid, cuttlefish and shrimp) to optimise PCR
142 conditions. Multiplex identifier (MID) tags were added to the primer sequences and used to
143 assign DNA sequences to their respective samples (n = 34). MID tags were added to each of
144 the three tested primer sets (fish, cephalopods and crustaceans). For each of the three primer
145 sets we used 24 forward primers/MID and 2 reverse primer/MID combinations, and all *in*
146 *vitro* testing was performed using primer pairs first without and then with the MID tags to
147 ensure amplification was not affected.

148 To amplify DNA from fish and cephalopods, we used primers targeting the 16S region
149 originally published by Waap (2015) and modified from Chord_16S_F/Chord_16S_R
150 (Deagle et al. 2009). We further modified the primer sequence to comprehensively match the
151 range of potential prey species (Table 3). To amplify fish DNA, the forward primer has
152 additional CT bases at the 3´end for NGS sequencing to improve the blocking probes (see
153 below), so that the mismatch was not located at the last base pair (Waap, pers comm.). To
154 amplify cephalopod DNA, we modified the forward primer by one base and the 5´end of the
155 reverse primer. Both primer pairs tested positive *in silico* and *in vitro* for potential prey of red-
156 throated divers.

157 To amplify crustacean DNA, a primer combination targeting the Cytochrome oxidase I region
158 (COI) was used that was likely to amplify crustaceans and molluscs (Stockdale 2018, Table
159 3). The forward primer (Leray et al. 2013) was designed to amplify arthropod DNA, including
160 crustaceans and molluscs. The reverse primer (Simon et al. 1994) was also designed to
161 amplify arthropods including crustaceans. The primers tested positive *in silico* and *in vitro* for
162 potential prey of red-throated divers and provided a good coverage of our target species and a
163 good coverage with reference sequences available in public databases. This primer set

amplified a product size of 332 bp and thus represents a good compromise as it is long enough to provide good taxonomic information and short enough to survive digestion.

Blocking primer

The primers chosen to amplify fish prey were universal chordate primers that could also amplify other chordates, including predator DNA. To prevent the amplification of predator DNA, we developed a blocking probe using a C3 spacer (Table 3; Vestheim and Jarman 2008). However, the blocking probe reduced amplification success and a second amplification of samples was performed excluding the blocking probe. Gel electrophoresis (see below) was used to visually monitor the amplification of predator and prey DNA, assisted by the inclusion of red-throated diver (300 bp) and fish (264 bp) reference samples. This differential in PCR product size allowed for predator amplicons to be easily identified (Fig. 2).

PCR amplification of DNA from faeces

PCR amplifications were performed in single reactions using Multiplex PCR Kits (Qiagen) and a 20 µL PCR reaction volume. Thermal cycling conditions for fish and cephalopod prey were 95 °C for 15 min followed by 45 cycles of: 94 °C for 30 s, a primer specific annealing temperature (Table 3) for 90 s, and 72 °C for 45 s, followed by a final extension at 72 °C for 5 min. Thermal cycling conditions for crustaceans were 95 °C for 15 min followed by 45 cycles of: 94 °C for 30 s, a primer specific annealing temperature (Table 3) for 90 s, and 72 °C for 90 s, followed by a final extension at 72 °C for 15 min.

All PCR products were visualised by gel electrophoresis on 2% agarose gels stained with SYBR®Safe (ThermoFisher Scientific, Paisley, UK) and compared to a standardised 1000 bp ladder. The PCR product concentration in successful reactions was quantified with a Qubit fluorometer (Thermofischer) and subsequently pooled into two equimolar libraries of individually tagged amplicons (PoolA using a blocking probe and PoolB without a blocking probe). To remove primer dimer we ran a magnetic clean up (AMPure). Concentrations of DNA and primer dimer were measured on a tape station (D1000 Screen Tape; Tape Station Analysis Software A.01.05 SR1, Agilent technologies) and a Qubit before and after the magnetic clean up.

Next Generation Sequencing

NGS library preparations were performed at the NERC Biomolecular Analysis Facility – Sheffield (NBAF-S), Sheffield, UK using the NEBNext Ultra DNA Library Prep Kit for

Illumina (New England Biolabs, Ipswich, MA). To characterise the diet content of the individually tagged amplicons the libraries (PoolA and PoolB) were sequenced at the Sheffield Diagnostics Genetics Service (Children's Hospital, Sheffield, UK) using 250 bp paired-end reads on a MiSeq desktop sequencer (Illumina, San Diego, CA).

Bioinformatics

We performed eight steps to transform the raw Illumina sequence data into a list of molecular operational taxonomic units (MOTUs) with assigned taxonomy. These steps included assessing sequence quality, trimming sequences (Bolger et al. 2014), aligning paired reads (Magoc et al. 2011), matching sequences to MID tags and amplicon primers (Schloss et al. 2009), and demultiplexing sequences into files for each amplicon. We used USEARCH (Edgar 2010) to dereplicate the sequence file, to detect and to remove chimeric sequences and to cluster into MOTUs based on 97% identity. Clustering is an important step in metabarcoding analysis to group similar sequences into distinct taxonomic units, but remains one of the central challenges. If the clustering threshold is too conservative, e.g. 5% sequence divergence, the dietary richness could be underestimated due to a high mean overlap of MOTUs. Conversely, a less conservative decreased threshold, e.g. 2% sequence divergence, could overestimate species richness (Clare et al. 2016). Here we applied the established clustering threshold of 97% similarity (Edgar 2013, 2016) using the 'cluster_fast' function in USEARCH (Edgar 2010). We applied the BLASTn algorithm (Altschul et al. 1990) to match MOTU sequences to reference sequences in the NCBI GenBank nucleotide database, using a cut-off of 90% minimum sequence identity and a maximum e-value of 0.00001. For detailed information about options, parameters and values please see Table 1 in the supplementary material.

We subsequently manually performed further filtering steps to produce robust taxonomic assignments. We discarded MOTUs (sequence clusters 97%) that corresponded to contaminants that can occur regularly in faecal samples, such as bacterial, human or predator DNA. MOTUs were retained in a sample only if they contained a minimum of 5 sequences. Taxonomic assignment was based on the percentage similarity of the query and the reference sequences. Since short fragments are less likely to contain reliable taxonomic information we only retained sequences with a minimum length of 190 bp and a BLASTn assignment match greater than 98%, following Deagle et al. (2009) and Vesterinen et al. (2013).

Finally, we combined both pools (PoolA with a blocking probe and PoolB without a blocking probe) together for final analyses. To avoid overrepresentation we excluded prey species of samples from PoolB that were also present in PoolA.

Analysing the Blast output

We used MEGAN Community Edition version 6.8.8 to visualise the accession number identifiers on the NCBI taxonomy (Hudson et al. 2016). We imported the blast output and used the default LCA parameters to assign a taxon name to each MOTU (Huson et al. 2007). If all retained hits of a MOTU with the same quality criteria (sequence identity, sequence length, e-value) matched the same species then we have a species-level assignment, otherwise the MOTU was assigned to the lowest shared taxonomic level, e.g. genus or family.

Statistical analysis

We analysed prey range by determining the presence of prey items, their frequency of occurrence (FO) (Barrett et al. 2007, Tollit et al. 2009), and species richness. FO was calculated as: FO = (n/t) X100 where n was the number of samples in which the specific prey item appeared and t was total number of samples containing prey. FO reveals the percentage of sample units in which each prey item occurred (Barrett et al. 2007). The number of MOTUs (defined by 97% clustering threshold, n = 169) assigned for each prey taxa were additionally presented as percent occurrence in faecal samples (n=29) to visualise the sequencing output Fig. 4. No further quantitative analyses were done with these data due to a range of possible biases and as interpretation of sequence proportions generated via high-throughput sequencing requires careful data analysis (Deagle and Tollit 2007; Pompanon et al. 2012; Deagle et al. 2013, 2018).

Whether or not there is consistency in prey consumption by red-throated divers over time informs our understanding of prey selection in this particular area. We tested this by comparing FO of prey items in 13 samples from 2015 with FO of prey items in 16 samples from 2016. Statistical tests suitable for small sample sizes were performed in Rcmdr (Fox and Bouchet-Valat 2018). We used Pearson's chi squared-test to compare the frequency of occurrence between years for each prey group when sample sizes fulfilled the minimum requirements for this test (n >5). When sample sizes were small (n < 5), we implemented the Fisher's Exact Test for Count Data. To compare the number of prey detections per sample between sampling years the T-Test for independence was used. Small sample sizes precluded further analyses (e.g. comparing seasons) or to use other statistical tests. Considering the

sample size and the temporal scope of faecal DNA sampling only marked differences were expected to be identified.

Results

Overview of sample quality and prey species found

Neither cephalopods nor crustaceans were detected in the diet, despite successful *in vitro* PCR amplification using reference tissue samples from potential prey items from the German Bight (octopus, squid, cuttlefish and shrimp samples).

The fish primer set produced more than 800,000 sequences from both pools combined, for specific information on number of sequences during bioinformatics analysis, see Table 2 in supplementary material. Of 34 screened samples 29 samples gave positive PCR amplifications (PoolA: n = 21; PoolB n = 29). Both pools had ~50% of MOTUs assigned to prey fish (PoolA = 56%; PoolB = 48%), plus with other MOTUs being from the predator DNA (red-throated diver) and contaminants such as bacteria and human DNA (Fig. 3). Using the blocking probe, we still amplified predator DNA but the amount of MOTUs assigned to the predator was slightly lower in PoolA (9%) than in PoolB (17%).

After filtering for contaminants, sequence length and mapping to reference sequences, 20 and 24 faecal samples remained for PoolA and B respectively. After merging both pools, the final sample set consisted of 29 samples (PoolA n = 20, PoolB n = 9) which corresponds to 85% of all samples collected (Table 2). Four samples were discarded (PoolB) as they contained only contaminants and predator DNA, and two samples were discarded as the amplicon length criteria were not met (1x PoolB, 1x PoolA).

Clustering the sequences by 97% similarity to each other and subsequent filtering resulted in 169 MOTUs that were used for further analyses. A list of a representative query sequences of each MOTU and its quality criteria is listed for each prey assignment in Appendices (Table A1) and for all MOTUS in Table 3, supplementary material. For the two sampling periods 19 taxa from 13 families were identified in 29 faecal samples (Fig. 4, Table 4). In 2015 we detected a slightly higher number of taxa in comparison to 2016 (18 and 13 taxa assigned to species, respectively; Table 4). The prey species spectrum was similar between the two years with 12 matching taxa and no significant differences ($\chi^2 = 1.004$, $p = 0.316$). European anchovy (*Engraulis encrasicolus*), turbot (*Scophthalmus maximus*), European pollock (*Pollachius pollachius*), cod (*Gadus sp.*), European bass (*Dicentrarchus labrax*) and sand

289 lances of the genus *Ammodytes* were detected only in 2015, and whiting (*Merlangius*
290 *merlangus*) only in 2016 (Table 4).

291 *Prey detection*

292 Of the samples where prey were detected, the mean number of taxa found was 4.2 ± 0.7 per
293 sample (n=29) with minimum and maximum values of 1 and 16 respectively. There was no
294 significant difference (t = 1.58, p = 0.135) between the number of prey items detected in 2015
295 (mean = 5.3) and 2016 (mean = 3.1).

296 Clupeids were the most frequently detected prey group (FO of 65.5%, Table 4). Within this
297 group, Atlantic herring (*Clupea harengus*) and European sprat (*Sprattus sprattus*) occurred
298 most frequently (FO of 55.2% and 58.6%, respectively). No significant differences were
299 found between years for clupeids ($\chi^2 = 0.030$, p = 0.863), European sprat ($\chi^2 = 0.283$, p =
300 0.595), or for Atlantic herring ($\chi^2 = 0.005$, p = 0.945).

301 The Atlantic mackerel (*Scomber scombrus*) was the only species of mackerel detected (Table
302 4), with a total FO of 55.2% and no significant differences between the two sampling years
303 (FO 53.8% in 2015, FO 56.3% in 2016; $\chi^2 = 0.005$, p = 0.945).

304 Flatfish were recorded with a total FO of 51.7% (Table 4) and no significant difference
305 between the two sampling years (61.5% in 2015, 43.8% in 2016; $\chi^2 = 0.287$, p = 0.592). Most
306 taxonomic assignments were at the family or genus levels. Righteye flounders
307 (Pleuronectidae) were dominant and where MOTUs were assigned at the species level the
308 common dab (*Limanda limanda*) was the most frequent species detected.

309 Gadoids (Gadidae) were recorded with a total FO of 37.9% and high similarity between
310 sampling years (38.5% in 2015, 37.5% in 2016; $\chi^2 = 0.001$, p = 0.972, Fishers exact test p =
311 0.976). Most MOTUs could only be assigned to the family level, but of those assigned to
312 species cod (*Gadus sp.*), European Pollock (*Pollachius pollachius*), whiting (*Merlangius*
313 *merlangus*) and haddock (*Melanogrammus aeglefinus*) were detected at least once. Detections
314 of these species varied between years but sample sizes were too small for statistical tests.

315 Sand lances had a total FO of 31%, with a similar proportion of greater sand eel (*Hyperoplus*
316 *lanceolatus*; FO of 13.7%) and sand lances of the genus *Ammodytes* (FO of 20.7%). There
317 were significantly more sand lances detected in 2015 (61.5%) in comparison to 2016 (6.3%;
318 $\chi^2 = 5.394$, p = 0.020; Fishers exact test p = 0.026).

Other prey species infrequently occurred and are detailed in Table 4 and Figure 4.

Discussion

The aim of this study was to analyse prey species composition in faecal samples from red-throated divers caught in the German Bight, using high throughput sequencing. In our data set we found an exclusively piscivorous diet, with no evidence of cephalopod or crustacean consumption and a similar prey spectrum between two consecutive sampling years.

Application of high throughput sequencing to study diver diets

The DNA metabarcoding methodologies utilised in this study have previously been applied in diet studies on other marine predators (Deagle et al. 2005, 2007; Pompanon et al. 2012). However, this study is the first application of this approach to analyse the diet of red-throated divers in the German Bight or elsewhere. Using reference sequences, we found high taxonomic coverage for both the COI and 16S barcode primers. Because of their commercial importance in the German Bight many fish species (e.g. Atlantic herring), alongside some cephalopod species, are well studied and the majority of these species appear in the Genbank database (Dickey-Collas et al. 2010, Engelhardt et al. 2013).

Sequences were clustered at 97% identity and represented consistent taxonomical units (MOTUs). Some prey species were represented by multiple MOTUs, suggesting that the clustering threshold could have been lower. However, a lower threshold would have increased the risk of clustering two closely related species into a single MOTU and thus reduced taxonomic discrimination. In practice, it is difficult to apply an 'average' threshold when diet is diverse and the prey are likely to have differing evolutionary rates. On balance, we deem the clustering threshold applied as appropriate and this method provided a good estimate of species richness with distinct taxonomic units.

We obtained sufficient sequencing data from 85% of the analysed faecal samples using universal primers. The species richness was higher in 2015 but individual variances may be due to sampling conditions, sample quality and amplification success. The use of a blocking probe proved to be of little advantage, with sufficient prey DNA amplified using both approaches (Fig. 3). The use of a blocking probe reduced the amplification of predator DNA but also amplification success in general since the output of prey-positive samples was higher when the blocking probe was omitted.

The detection rate of prey species can be biased by the method applied. For example, Tollit et al. (2009) found some prey (Ammodytidae, Cottidae and Gadidae) were more reliably detected with morphological tools, whereas other prey (Salmonidae, Pleuronectidae, Elasmobranchii and cephalopods) were only detected with molecular tools. However, the overall results did not dramatically differ. In general molecular methods have been shown to identify more trophic links (number of taxa identified) with higher rates of taxonomic discrimination in comparison to morphology (e.g. Soininen et al. 2009; Alonso et al. 2014; Berry et al. 2015; Waap et al. 2017). Using molecular methods, we found a similar prey composition to conventional morphological methods applied in previous studies on red-throated diver diet. Using faecal samples coupled with DNA metabarcoding is now an established non-invasive approach for dietary studies. However, it is debatable whether or not this method can provide quantitative (read number) in addition to qualitative (presence and absence) estimates of diet (Deagle and Tollit 2007; Pompanon et al. 2012; Deagle et al. 2013; 2018). In this study we applied a conservative approach of using only qualitative data. However, if quantitative data are required we recommend combining DNA metabarcoding and morphological methodologies, where the latter can provide quantitative information as in Alonso et al. (2014) and Waap et al. (2017).

A faecal sample, for most species, will represent an individual's most recent meals. Other methods, including fatty acid composition and stable isotope analyses, can provide information over a longer time frame (Meier et al. 2017). Although our sample size is small, samples were collected from birds caught in two consecutive years at dispersed intervals encompassing late winter and spring (February – April); when red-throated diver abundance is highest in the German Bight. Thus, this dataset provides dietary information from a time period when this area is particularly attractive to these birds. Wintering home ranges of red-throated divers can cover several connected sites, including sites outside the German Bight, such as the Baltic Sea (Kleinschmidt et al. unpub data). The German Bight also represents an important staging area in spring when some birds have already started migration (Garthe et al. 2015) and the availability of suitable prey types is probably one of the main determinants of habitat quality for these birds. In this context the time frame over which a faecal sample provides dietary information helps to reflect the situation in the particular area of interest for this study.

Fish availability in the German Bight, red-throated diver diet and comparison to previous studies

382 Potential prey availability is an important factor affecting habitat choice and diet selection.
383 We searched the species factsheets (ICES 2006 a,b), reports and publications (ICES 2008,
384 2011, 2016, 2017a, 2017b, 2018; DFS 2016) to compare fish distribution (a proxy for
385 potential prey availability) with the diet of red-throated divers in our study in addition to
386 previous studies. In our dataset red-throated divers consumed a wide range of fish prey
387 species consisting of both a pelagic and a benthic component. We found mainly clupeids,
388 mackerels, flatfish, gadoids and sand lances in the diet of red-throated divers but no clear
389 dominance of a single species or species group could be identified. A similarly wide, although
390 slightly different range of prey species was found in previous studies on red-throated diver
391 diet. For example, Madsen (1957) found a broad prey spectrum but the majority of analysed
392 birds (82%) fed exclusively on cod, gobies, sticklebacks and herring with varying intensities.
393 Guse et al. (2009) found 11 species from 9 families with clupeids, zander, European smelt,
394 ruffe, lesser sandeel, three spined stickleback and common goby being dominant species.
395 Similarly, Durinck et al. (1994) identified clupeids and gadoids as the most frequent prey
396 items.

397 Clupeids, specifically sprat and herring occurred most frequently in both sampling years of
398 our study. These species are typically high in lipid content and energy density (Pedersen and
399 Hislop 2001; Ball et al. 2007). Sprat and juvenile herring are also two of the most abundant
400 pelagic species in the German Bight in spring (ICES 2006 a,b), which coincides with our
401 sampling period. The size of available prey fish is also important for prey selection. In
402 general, herring occurs in the North Sea with a size of 20-30 cm but in our sampling period
403 smaller (juvenile) herring with a size <20 cm are the most abundant and widely distributed in
404 the German Bight and the Kattegat (ICES 2006 a; Trueman et al. 2017). Sprat is a pelagic
405 species abundant in frontal areas of the North Sea with a size of <16 cm (Kanstinger and Peck
406 2009). We also found European sardine (*Sardina pilchardus*) and European anchovy
407 (*Engraulis encrasicolus*) in the diver diet but less frequently, which is consistent with the
408 distribution of both these clupeid species. They originate from the Mediterranean Sea (Motos
409 et al. 1996) and since 2003 are expanding into the North Sea (Kanstinger and Peck 2009).
410 Like sprat, sardine occurs in frontal areas whereas anchovy is primarily found in near-shore
411 areas. The distribution of clupeids is in good agreement with red-throated diver distribution,
412 which appear to be attracted by frontal zones (Skov and Prins 2001; Goyert et al. 2016;
413 Heinänen et al. unpubl data). Hence these areas provide a source of energetically valuable
414 species for red-throated divers. The high detection rate of clupeids is in line with two earlier

studies on red-throated diver diet and reinforces their importance as red-throated diver prey (Durinck et al. 1994; Guse et al. 2009).

Atlantic mackerel is widespread throughout the North Sea and is one of the most commonly exploited species (ICES 2011, 2016, 2017). Due to its high energetic value, mackerel is an attractive fish for seabirds (Montevecchi et al. 1984, 1988; Garthe et al. 2014). Overfishing triggered a population collapse in the North Sea in the 1970s but since 2000 the stock has increasing again (ICES 2011; Jansen 2014; Jansen and Gislason 2013; Jansen et al. 2012a, 2012b; 2014; Kooij et al. 2016). These changes in mackerel availability may explain why both Madsen (1957) and the current study detected mackerel in the diet, while Durinck et al. (1994) did not. Mackerel appeared in our samples in considerable numbers indicating that it may now be a more important prey than previously thought.

Most flatfish were identified to family level, but of those identified to species level, common dab was the most common in both years. Flatfish have been recorded in low numbers in red-throated diver diet (Madsen 1957; Durinck et al. 1994; Guse 2009), possibly due to their wide-bodied shape making adult flatfish an unfavourable prey item (Reimchen and Douglas 1984; Guse et al. 2009). Dietary studies in the adjacent Wadden Sea have shown that juvenile flatfish are selected as important food items by other water birds such as benthic feeding cormorants (Nehls and Gienapp 1997). The Wadden Sea and adjacent waters are an important nursery ground for several flatfish species (DFS 2016) and juvenile common dab is highly abundant in spring within the German Bight over a wide depth range (Beek et al. 1989; Bolle et al. 1994; Campos et al. 1994; Hufnagl et al. 2013; DFS 2016; ICES 2017a,b). Prey size cannot be deduced from metabarcoding but red-throated divers may be preying on juvenile flatfish. Although flatfish are considered to have a low energy content (Ball et al. 2007), the probable high encounter rate may explain the high detection rate in our samples.

Gadoids, particularly cod, were described by Madsen (1957) as the most important prey group for red-throated divers in the Kattegat and Belt Sea. In the current study, gadoids were infrequently present in the diet. This is in line with findings of Durinck et al. (1994) from the south-western part of the Skagerrak. Juvenile gadoids (<20 cm) are more likely than adults to be prey for red-throated divers. Recordings of this size class of gadoids are mostly restricted to the eastern inshore water of the Skagerrak and Kattegat, with low abundances in the German Bight (Munk et al. 1999, Munk 2014; André et al. 2016). Thus, gadoids may be a favoured prey item but low availability at the study site limits feeding on these species.

Sand lances are an important prey for seabirds in general, particularly in the North Sea (Harris and Wanless 1991; 2013; Mendel et al. 2008; ICES 2011; Engelhardt et al. 2013; ICES 2016). Sand lances appeared at a high frequency in 2015 but were less common in 2016 in our data set. This pattern is reflected in commercial catch rates for sand lances in the central and south-eastern North Sea ecoregion (Division 4b-c): average catch rates and a low recruitment in 2015 and low catch rates and high recruitment in 2016 (ICES 2018a,b). Previously, sand lances have been recorded at both high (Guse et al. 2009) and low (Madsen 1957, Durinck et al.1994) frequencies in red-throated diver diet. These patterns suggest that the frequency of sand lances in the diet is determined by their availability.

Smelt (*Osmerus eperlanus*) was not detected in this study but has been highlighted as an important prey species for red-throated divers in the Baltic Sea (Žydelis 2002; Guse et al. 2009). Smelt occurs in parts of the Wadden Sea with low salinity and close to the coast. Here it forms dense spawning aggregations in estuaries and anadromous migrations in late winter and early spring (DFS 2016). The German Bight is further away from river mouths, the lack of smelt in our dataset could probably be explained by the low abundance of this species here.

Sea trout (*Salmo trutta*), European hake (*Merluccius merluccius*), sticklebacks (*Gasterosteus sp.*), European bass (*Dicentrarchus labrax*) and sand goby (*Pomatoschistus minutus*) were recorded in our dataset at low frequencies. These species are widely distributed in the North Sea with varying densities. Some, such as gobies, are known to be important prey items for other marine predators (Haelters et al. 2012; Méheust et al. 2015; Andreasen et al. 2017) and were previously recorded as prey items of red-throated divers (Madsen 1957, Durinck et al. 1994, Guse et al. 2009). Sticklebacks were frequently found in all previous studies. However, the current study suggest that these species are of low importance for red-throated divers in the German Bight.

In contrast to our study, Guse et al. (2009) found zander as one of the most important prey items of red-throated divers wintering in the Baltic Sea. This fish species prefers freshwater or brackish habitats, and therefore is almost absent in the saline waters of the German Bight.

Non-fish prey such as insects, polychaetes, molluscs or crustaceans were detected in small amounts in all previous studies. Cephalopods were detected in a single previous study (Durinck et al. 1994). We found no evidence that non-fish prey were consumed by red-throated divers in the German Bight and thus our results reinforce previous conclusions that these taxa are not an important part of the diet.

In summary, prey species of red-throated divers identified in this study occur in the study area as both adult (e.g., clupeids, sand lances) and juvenile fish (e.g., gadoids, flatfish, mackerels). Thus the area seems to be a good foraging ground for red-throated divers. There is an overlap between the prey fish of red-throated divers and commercial fish species, like herring and mackerel (ICES 2011, 2016, 2017). This overlap increases the risk of gill-net mortality, which is a conservation issue in other regions such as the Baltic Sea. In the German Bight, there is a lower potential for such conflicts because trawls are more commonly used to fish as opposed to gill-nets. The oceanographic conditions (sea surface temperature (SST), salinity and chlorophyll a, NAO) were similar between the two sampling years and no important changes in prey community can be expected within such short timeframe, with the exception of the observed fluctuations in sand lance abundance. For this prey group, detections in the diet and reported catch rates (ICES 2018a,b) showed a similar trend. Reasons for this are unclear but sand lance productivity in the North Sea is known to fluctuate. Such fluctuations depend on a combination of several regulating factors including fishing, climate effects, density dependence and food availability (Wright et al. 2017; Lindegren et al. 2018). Although we present data from only two sampling years, the consistent pattern of prey species suggests a relatively stable diet that is likely to reflect the availability of these fish species in the study area. There are long-term increases in sea temperature and species usually associated with warmer waters are expanding their range to include the North Sea. Such species include European sardine and European anchovy (Kanstinger and Peck 2009). The diet of red-throated divers in the German Bight includes these expanding species and also recovering species like mackerel, indicating that the dietary data may reflect changes in the fish community and some flexibility in prey consumption. However, a larger sample size across a broader temporal scale is required to fully support this conclusion.

The samples analysed here were collected in late winter and early spring, shortly before the migration to the breeding grounds. For non-breeding red-throated divers little is known about energy expenditure, resource partitioning and energy requirements during wintering, staging and migration. Schmutz (2014) suggested that marine conditions could affect adult survival of red-throated divers with indications of a higher risk of mortality during the non-breeding season. Red-throated divers are medium sized birds with weight varying between 1400g – 2000g (own observations), and with high wing loading (Storer 1958; Lovvorn and Jones 1994). Despite this, these birds often need to cover long distances to their breeding grounds (www.divertracking.com; McCloskey et al. 2018), with some individuals travelling as far as 850km or 1300km in a single flight (Kleinschmidt et al. unpubl data). Weber et al. (1997)

showed the importance of resting sites for refuelling. Consequently, migration represents periods of high energetic demand and adequate energy reserves seem to be essential. If prey of rich calorific value becomes unavailable due to displacement effects, red-throated divers may fail to balance their energy budgets. In general, these birds winter in temperate marine waters with low ambient temperatures, consequently reliable and sufficient energy intake is likely to be a necessity and influences prey consumption.

Conclusion

Overall, our results demonstrate that the use of faecal samples coupled with DNA metabarcoding and NGS is a valid and appropriate approach to non-invasively study the diet composition of red-throated divers.

Our results provide important dietary data for red-throated divers in the German Bight, which is needed for a good understanding of their habitat preferences during wintering and spring staging. This baseline information can be used to evaluate changes associated with human developments in the offshore environment, changes in oceanography, or population declines. The results for the German Bight complement other dietary studies on red-throated divers that show a somewhat different composition of fish species, reflecting regional differences in fish fauna. Among a generalised prey spectrum, bentho-pelagic schooling fish seem to dominate the diet of red-throated divers (Cramp and Simmons 2004; Guse et al. 2009). In our study five species groups are concluded to be major dietary components for red-throated divers in the German Bight. We found clupeids, mackerels, flatfish, and gadoids occurring in substantial proportions in both sampling years, and the frequency of sand lances varied between the two sampling years. Hence the diet consistently includes some common species with a high nutritional value (Hislop et al. 1991; Ball et al. 2007), indicating the importance of these fish groups as prey items for red-throated divers in the German Bight. Red-throated divers stage in a specific habitat, mostly influenced by frontal zones in coastal areas in the German Bight (Skov and Prins 2001; Heinänen et al. unpubl data). The preferred feeding at frontal zones may also explain the higher abundance of pelagic fish among the red-throated diver prey, where these species aggregate, while demersal species depend mainly on suitable sediments. Considering the effects of disturbance, displacement or barrier effects arising from anthropogenic activities such as ship traffic and offshore wind farms (Mendel et al. 2019), the broad prey spectrum that we found could indicate resilience of red-throated divers against changes in community composition of available fish or resilience against displacement from suitable habitat. However, if alternative sites of high-quality habitat are not sufficiently

546 available, displacement may result in a decreased energy intake and subsequently poorer body
547 condition. Thus, altered food accessibility as a result of disturbance or displacement could
548 have severe effects on red-throated divers. In general, the availability of some prey species
549 may explain, at least to some extent, the preference of this area as wintering and staging
550 habitat. Further studies could aim to discern whether the birds use this area because of a high
551 abundance of suitable and energy rich prey or if they simply feed on the most abundant prey.

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566 Compliance with ethical standards

567 Conflict of interest: The authors explicitly declare that they have no conflict of interest.

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